Maximizing Cell Growth per Passage Using Polystyrene Fibers

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Abstract

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Nazmiye Celik
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Tissue-engineering strategies often demand a large number of stem cells and cells passaging, which is usually achieved cell expansion on a two-dimensional (2D) substrate, such as a Petri dish or culturing flask. Stem cells on these 2D systems become confluence in about one or two weeks, reaching 3 to 5-fold maximum proliferation, and must be trypsinized, detached and re-plated to continue cell expansions. However, trypsinization exposes stem cells to biochemical and biophysical stresses that are known to accelerate cell senescence, phenotype changes, and the loss of stemness and multipotentiality. In this study, I hypothesized that a cell-expanding strategy that maximize the folds of cell proliferation before trypsinization may promote the phenotype stability of stem cells. A three-dimensional (3D) cell-expansion system using ribbon-shapes polystyrene was developed, which is the same material used for making culture flasks. In contrast to traditional cell expansion, these micro ribbons (500 micron wide and 50 microns thick) provide a gradually increasable surface area per weight to support high cell proliferation, enabling a maximum cell number per passage that is higher than the traditional system (by more than 10 folds). Cells reaching confluence were re-suspended by trypsin following the similar protocols for the 2D cell expansion. Human mesenchymal stem cells (hMSCs) were used as the model cell type to compare the differences between the results of my 3D cell expansion and that of the 2D cell expansion. These results include the MSC proliferation rate on, change of gene expression post passaging on the both 3D ribbon-based and 2D traditional groups. In addition, the capacity MSCs expanded by traditional 2D method to differentiate into bone and fat-like cells phenotypes was evaluated with increasing re-plating and passaging the cell culture. The result of my study showed that the PS-ribbons provide a 3D environment for cell expansion with enabling continual cell proliferation, in the absence of the traditional cycles of trypsinization, cell

detachment, and re-plating cycles. With this study, the efficacy of using PS ribbons was evaluated in terms of the performance of cell proliferation rates, and the maintenance of MSC phenotype.

1. INTRODUCTION

1.1. Limitation of the Existing Platforms for Cell Expansion

During the past 100 years, researchers have been continually inventing the suitable substrates for stem cell culture and expansion [1]. Glass surface, plastic surface, and treated polystyrene surfaces are commonly used for cell culture platform [2], alone with surface coating that promotes stem cell attachment and cell proliferation [3]. The surface area of these cell culture substrates put an upper limit to the maximum number of cells that can grow [1, 4]. Stem cells proliferate on these substrates and usually become crowded (or confluence) in about one or two weeks [5], reaching 3 to 5 folds maximum proliferation [6], and require cell passaging to enables further cell expansion and obtain the needed cell number. A typical polystyrene substrate, for example, supports a maximum cell density of 20,000 to 40,000 cells per cm^2. The total yield of stem cells from each passaging depends on the total surface area of the substrate. The typical cell yield from a 175 cm^2 cell-culturing flask, for example, is about 4 to 7 million cells per passage. If a larger cell number is desired, the stem cells must be passaged for continual expansion, in which cells from each culturing flask/plate are detached, divided, and re-plated to new culturing flasks/plates. However, the above cell detachment and re-plating procedure can inflict biochemical and biophysical stresses that accelerate cell senescence and decrease the stemness of stem cells (the differentiation potential of the stem cells) [7]. Re-plating procedure after serial passages in the cell culture causes the loss of multipotentiality [8, 9]. Most of cell detachment solutions such as trypsin cause loss of cell surface proteins [6]. The yield of cell expansion per passage may be maximized by minimizing the initial cell density. Nevertheless, overly low cell seeding density (<5,000 cells per cm^2) may induce cell death or cause extremely slow proliferation, due to insufficient of cell-cell signaling that promote cell growth and survival [10, 11]. Given the above limitation, the question I have when starting this research project was: “what if the cell-culturing surface itself can be continually increased overtime, as the cells number grows?”

In the engineering systems that demand large surface area, such as heat exchangers, maximized surface area per volume is often achieved by using a porous substrate made of foams, fibers, or ribbons made of shredded metals. My project was to explore the feasibility and efficacy of applying such principle to cell culture, using a porous material, made of polystyrene ribbons, as the substrate to enable a user to gradually increase the amount of surface area for cells expansion as the cells continue to proliferate. This new platform may simultaneously allow a standard high initial seeding density (>5,000 cells per cm^2) while providing a larger surface area for cell proliferation. My hypothesis was that such porous, three-dimensional (3D) substrate for cell culture can maximize the folds of cell proliferation per passage, minimize the requirement for cells passage, and promote the phenotype stability of stem cells by reducing or eliminating the cycles of cells re-planting.

1.2. Increasing Surface Area-Weight Ratio by Using Fibrous or Ribbons-like Substrates

Polystyrene was chosen as the material to synthesize ribbon-like substrates for my 3D cell expansion. Polystyrene (PS) has been established as a standard material for cell culture, for PS is a thermal plastic polymer that is manufacturing friendly, biochemically stable, and can be easily modified to enable the adhesion of cells and cell-produced extracellular proteins. Disposable Petri dishes and culturing flasks, which are the standard platforms for cells expansion, are usually made of PS [1].

The experimental scheme is shown in Figure 1. Substrates formed by fibers or ribbons-shape polystyrene allow the stem cells to be initially seeded at the bottom of a cell-culture flask by the standard seeding density, but provide a large surface area, alone the vertical direction, that is many-folds larger than the bottom surface. Different from the conventional PS dishes and flasks that have a limit surface area to support cell proliferation in the horizontal direction, fibers and ribbons-shape PS provide a three-dimensional (3D) construct to allow the stem cells to migrate and expand in the vertical direction, resembling a “skyscraper” for the cells. Since new PS fibers/ribbons can be added to the cell-culturing flask to accommodate more cells proliferation after the

existing substrates become cells-crowded, such platform may provide a gradually increasable surface area for stem cells expansion, potentially enabling an extremely high yield per cell expansion. If proven successful, this approach may eventually eliminate the need for conducting the cycles of cell detachment and re-plating to reach any desired stem cells number.

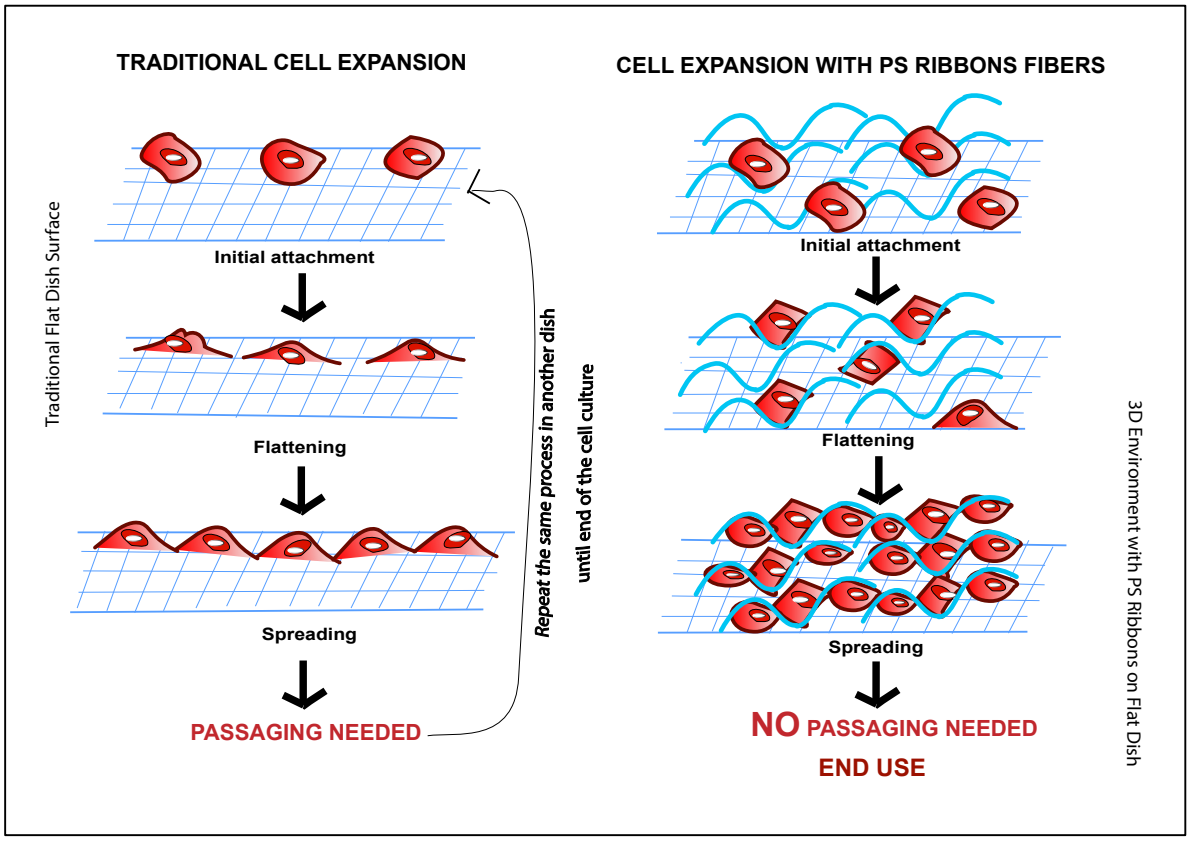


Figure 1. Cell expansion on PS ribbons versus traditional cell expansion

1.2. Goals of the Master Study

In this Master thesis, I had the following specific aims:

(1) Determine whether the use of fibers and ribbons-shape substrates for stem cell expansion can eliminate the needs for stem cells passaging to reach a desired cell number.

(2) Determine if this approach can improve the stability of stem cell phenotype and stemness, in comparison with the traditional 2D culture that demands constant cell detachment and re-plating.

(3) Prepare fibers and ribbons-shape PS substrates and examine the efficacy of using these substrates for cell expansion. Create a 3D cell expansion platform that which provides increasable surface area for cell growth.

(4) Make comparison between the outcomes of conventional stem cell expansion and the fibers/ribbons-based stem cell expansion. The outcomes include the cell proliferation speed, the achievable maximum cell number upon cells confluence, the needed cycles number of cell passaging, and the stemness of stem cells after cell expansion.

2. METHODS AND EXPERIMENTS

2.1. Production of Fibers and Ribbons-shape PS Substrates

To produce the 3D porous substrates from PS, I explore two potential fabrication methods to produce PS fibers and ribbons. The as-produced fibers and ribbons were tested by seeding with human mesenchymal stem cells and by examining the proliferation of MSCs.

2.1.1. Making PS fibers by Thermal Spinning, Using a Cotton-Candy Machine

Thermal spinning is a widely used industrial method to mass-produce thin fibers from bulk polymers. In my first attempt to fabricate PS fibers, a commercialized, 20'' carnival cotton-candy machine was purchased and used to achieve the thermal spinning (Figure 2). The cotton-candy machine consists of a spinning crucible to turn sugar or polymer into thin fibers. This crucible was electrically heated to above 200 ^{0}C, and had four side holes that served as nuzzles to jet the melted polymers. Upon melted, the polymer was pushed through the jet holes by the centrifuge force, forming thin fiber jets. A fan underneath the crucible generated the airflow to draw the jet of melted polymer away from the crucible while cooling the polymer melt back into the solid phase. Polystyrene fine powder (Goodfellow, Coraopolis) was mixed with sucrose (table sugar) at 1:10 PS/ sucrose weight ratio, and the mixture was used as the raw materials for the thermal spinning. I used sucrose as the carrier for the PS polymer fibers, since the melted PS polymer alone was too dense and did not provide the proper viscosity needed for forming thin fibers. Post spinning, PS fibers were retrieved from the bundle of as-spun fibers (which contained both polystyrene and sucrose) by removing the sucrose using water (Figure 2). The as-fabricated PS fibers were subsequently treated for cell adhesion, as will be explained in the later section.

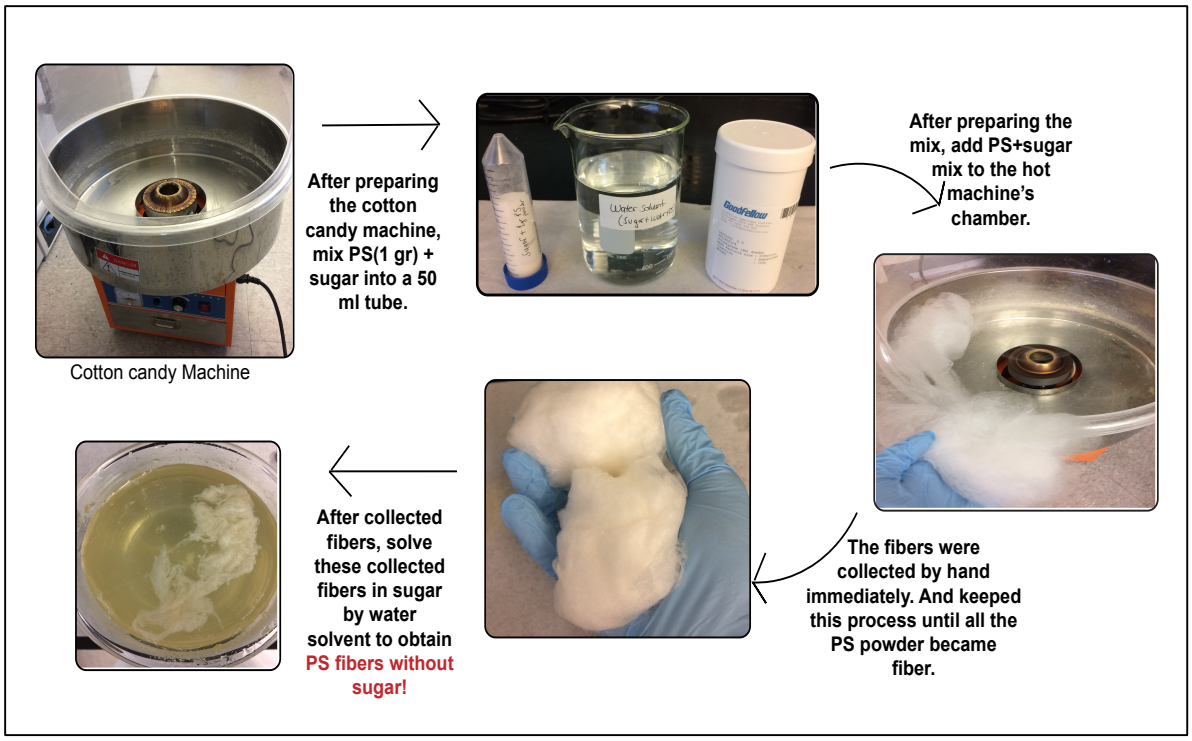


Figure 2. Producing of PS Fibers by using Cotton Candy Machine

2.1.2. Making PS Ribbons by shredding a Polystyrene Sheet

A potential drop back of thermal spinning is that the high temperature might damage the chemical property of PS and render the fiber non-cell adhesive. An alternative way to produce fibers/ribbons-like PS substrates is by directly cutting a thin sheet of PS film. In this approach, stacks of 50-micron thick PS films were purchased from Sigma Aldrich and were shredded into 500-micron wide PS ribbons using a paper cutter (Figure 3)**.** In comparison with the thermal spinning method, cutting PS ribbons from PS film was a slower method but could avoid the heat processing that might oxidize polystyrene.

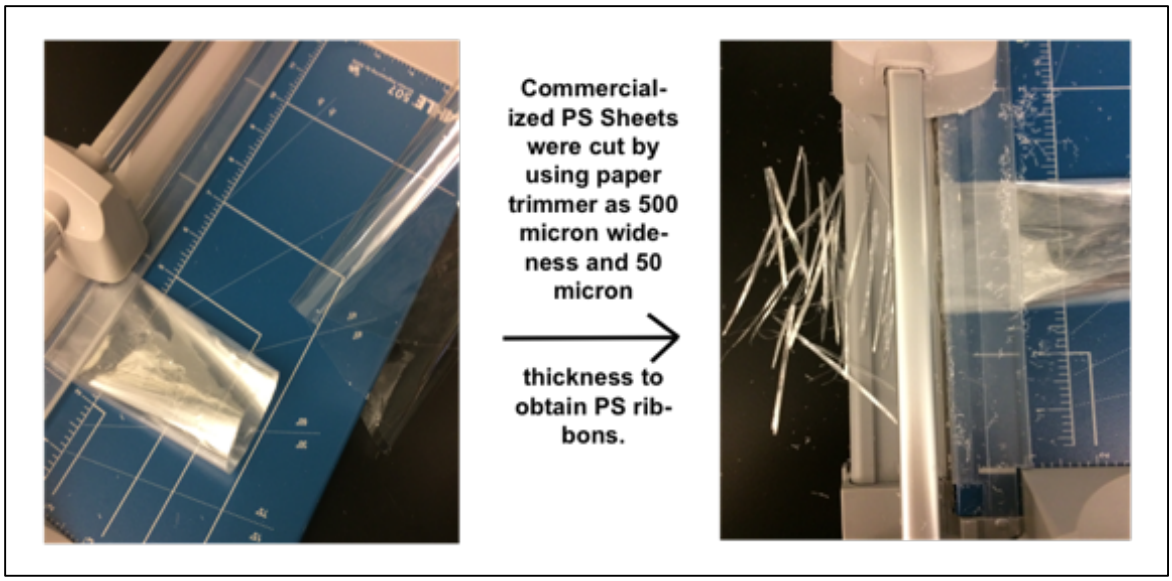


Figure 3. Preparation of PS Ribbons

2.2. Surface Treatments to Enable Cells Adhesion

To enable cell culture, the fibers or ribbons-shape substrates require chemical treatments to form surface ligands that facilitate stem cell adhesion. Such ligands include hydroxyl groups, sulfonic groups (-SH), and carboxylic groups (-COOH) [12, 13]. These groups increase the hydrophilicity and protein affinity of the polystyrene surface [3, 14, 15] , which are crucial factors for stem cells expansion [16].

The surface of original polystyrene consists of aromatic groups that is highly hydrophobic and has limited affinity to the cells' substrate-binding proteins and the extracellular matrix components (Figure 4). This surface property can be significantly improved by oxidizing the aromatic groups of PS by wet (acid, alkali), dry (plasma), and radiation treatments [17-22], which have been achieved by the following methods common used for the manufacturing of polystyrene-based lab wares:

- Water vapor plasma treatment,
- O_2 plasma discharge,
- Corona discharge,

- Adsorption of blood plasma protein and fibronectin on to the polymer surfaces,
- Acid treatments [20].

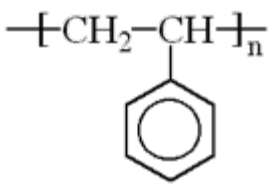


Figure 4. Chemical Structure of the Polystyrene

I chose acid treatment method, since it was the most effective and economic way for my lab. Among all kinds of acids, perchloric acid has been found to be one of the most efficient acid to oxidize polystyrene and introduce charged groups to the PS surface, such as hydroxyl (-OH) and carboxyl (-COOH) groups, which improves cells and protein adhesion [23]. The PS fibers and ribbons were perchloric acid-treated following the below protocol: 30 ml of 70% perchloric acid and 20 ml saturated aqueous potassium chlorate were mixed and the fibers and ribbons produced by the thermal spinning and cutting were submersed in the acidic solution for one hours, under constant stirring (Figure 5). The treated PS fibers/ribbons were washed by neutralized by sodium bicarbonate, rinsed by DI water, and dialyzed for three days to completely remove salts and acidic components from the PS surface.

2.3. Dialyzing and freeze-drying the PS Fibers/Ribbons

The acid-treated PS fibers/ribbons were submersed in 70% ethanol, which kept the fibers and ribbons sterile, sealed in dialysis bags, and wash for 3 days in tap water. The water was changed for nine times for three days in total and was changed three times for each day. After the dialyses, the fibers and ribbons were transferred to centrifuged tubes with sterile DI water, frozen under -80 ^{0}C overnight, and freeze-dried using a freeze dryer (Labconco Co.) under 50 mTorr vacuum at -50 ^{0}C for 3 days (Figure 5). The PS fibers and ribbons were stored under -20 ^{0}C before use.

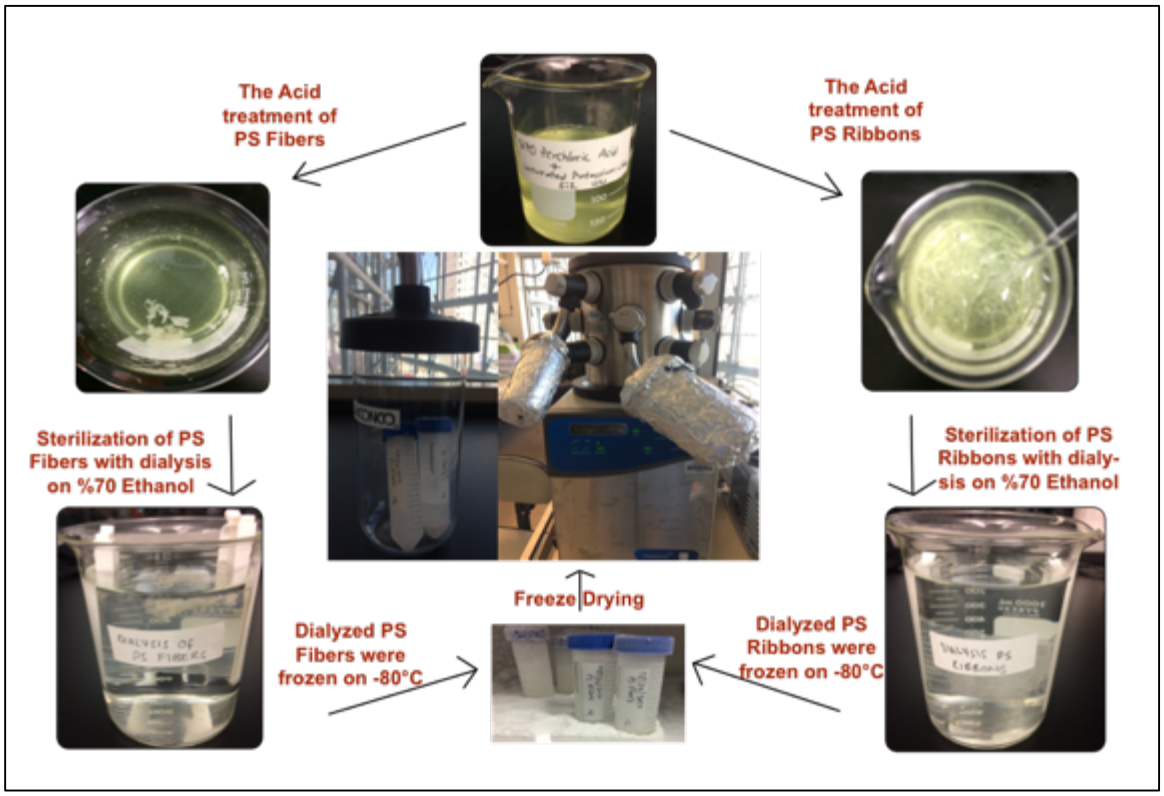


Figure 5. Treatment and Sterilization Method of PS Fibers and PS Ribbons

2.4. Culturing Mesenchymal Stem Cell on Fibers and PS ribbons and cell seeding

Before cell culture, the PS fibers and ribbons were protein-coated to increase the surface ligands to support cell adhesion. In brief, PS fibers and ribbons were soaked in the solution of bovine gelatin (0.01% in DI water) under 37 ^{0}C for up to 24 hours. Gelatin coating has been shown to significantly improve cell adhesion and proliferation, and was the regular treatment for stem cell expansion in my lab. The effect of time for gelatin exposure was examined according to the efficiency of cell adhesion and cell growth.

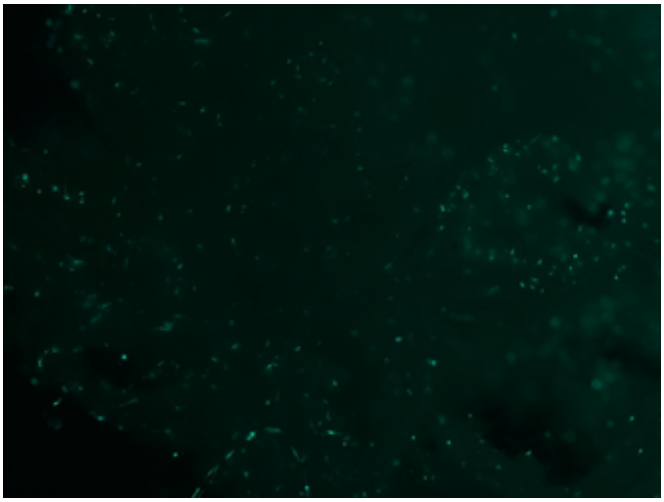


Figure 6. P-5 hMSCs on acid treated PS fibers after 1 week

Mesenchymal Stem Cells were selected as the model cells to examine the effects and efficacy of fibers and ribbons-shape substrates on cells expansion. Human mesenchymal stem cells (hMSCs) are adult stem cells found in some human tissues including bone marrow, synovial tissues, and adipose tissues. They have been shown to be capable of differentiating into bone, cartilage, muscle, and adipose tissue. [24] They can be used for regenerative medicine and in tissue engineering applications. Because of their multi-potent capabilities[25], MSC's can readily differentiate in culture depending on cytokine exposure, chemical exposure, or the change of environmental conditions, including force, pressure, and biophysical cues [26].

Human MSC was purchased at passage-2 (from Lonza). The cells were counted and seeded onto tissue culture flasks with Dulbecco's modified Eagle medium (DMEM), containing fetal bovine serum (FBS) (10%) and antibiotic penicillin/streptomycin (P/S) solution (1%). Following the initial 48 hours of incubation at 37 °C and 5% CO2, cells were washed with PBS and expanded in DMEM with 10% FBS, 1% P/S and 10 ng/mL basic fibroblast growth factor. The cells were passaged upon 85–90% confluence and passage-5 cells will be used for all experiments.

The gelatin-coated PS fibers or ribbons were loaded into cell culture dishes or flask (which provides 9 to 55cm^2 surface area for cell expansion) by about 1 gram/per cm^2.

The flasks were filled with hMSC culture media, and the hMSCs at passage-5 were seeded by 7000 cells per cm^2 into the flask, among the PS fibers/ribbons. Each culture was incubated at 37 °C under 5% CO_2 pressure. Culture media was changed every two days. The PS fibers/ribbons were briefly stirred once each day to enhance the distribution of newly divided hMSCs among the PS fibers/ribbons.

To prepare for a control group, the same types of cell-culture dishes and flasks were seeded with the same density of hMSCs (7,000 cells per cm^2), but were not added with any PS ribbons or fibers. The controlled samples were incubated with the same culture conditions. Cells in the control groups were trypsinized and re-plated once cell confluency was reached.

2.5. Failure of PS Fibers and Success of PS Ribbons

After 1-week cell culturing (culture media prepared by using protocol-2 was changed every 2 days), two repeated pilot studies showed that the PS fibers made by the cotton-candy machine were incapable of supporting the attachment (Figure 6), survival, and proliferation of hMSCs. One week after cell seeding, the average cell-density of hMSCs among the PS fibers one week after cell seeding was 1179.17 ± 210.42 cells per cm^2 (Figure 7). Most MSCs among the PS fibers remained spherical (Figure 6), and the number of live cells continually decreased, as shown by the decreasing signal from Calcein-AM staining (Figure 9b). In contrast, the pilot studies showed that the PS ribbons made by the direct cutting of PS thin film supported the attachment, survival, and proliferation of hMSCs (Figure 9a). One week after cell seeding, the average cell density of hMSC among the PS ribbons was 3823.21 ± 1425.12 cell density per cm^2 (Figure 7). Therefore, I dropped the use of PS fibers and selected the PS ribbons as my 3D platform for cell expansion.

The reason of why the PS fibers failed to support MSC proliferation is unknown at this point. Given the fact that the PS fibers were fabricated under an oxidizing temperature (>200°C), it is possible that the heat had destroyed the surface chemicals that were needed for cell adhesion or protein absorption. It is also possible that the use of sucrose as PS carrier had contaminated the PS.

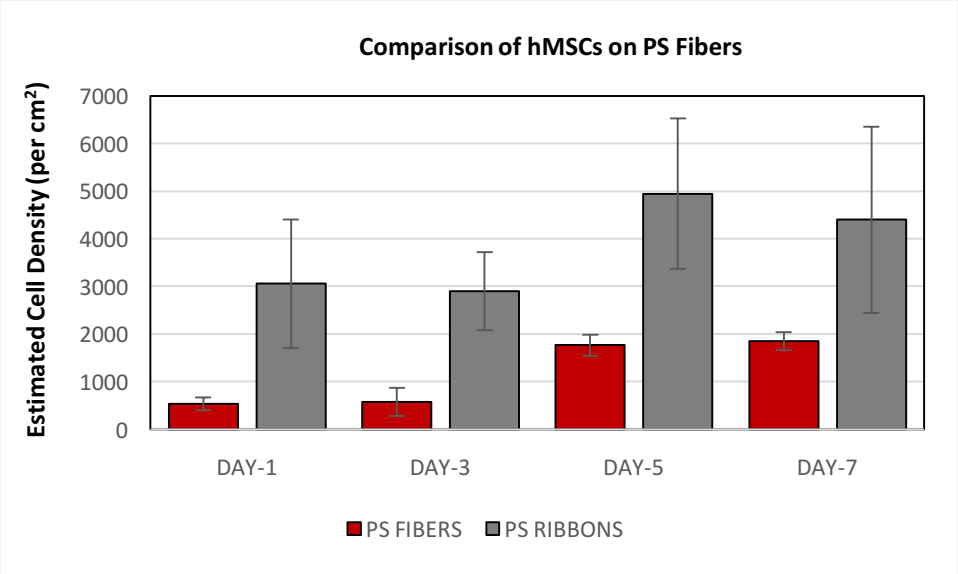


Figure 7. The change of cell densities among PS Fibers and PS Ribbons one week after cell seeding

2.6. Quantifying Cell Proliferation

For every two days, cell viability and proliferation was evaluated using Calcien-AM cell staining (Protocol 3), which labeled live-cells (ThermoFisher), and fluorescence microscope imaging. The change of cell density was estimated by sampling the PS fibers or ribbons from the culture flasks and counting the total number of cells on each ribbon. For each flask, the total surface area of the PS ribbons was calculated using the following numbers (Figure 7):

d=density of PS (g/cm^3)

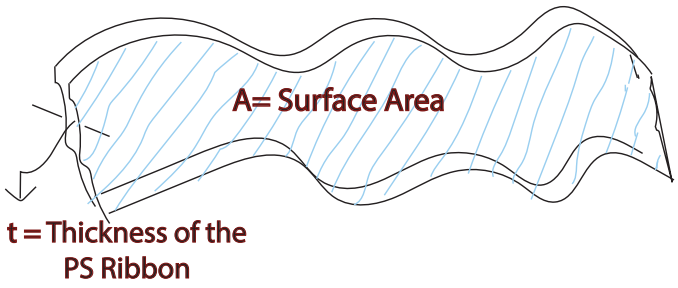
W= weight of ribbons (g)

t= thickness of PS ribbons (cm)

v= volume of total PS (cm^3)

A = Surface area

2A= Total surface area



$$\frac{W}{d} = v\,, \qquad A = \frac{v}{t} = \frac{W}{d.t}$$

Figure 8. Calculation of cell density on the PS Ribbons (t=0.005 cm, d=1.05 g/cm^3)

The total surface area of the PS ribbons in the cell culture dishes were calculated as shown in Table-1. To quantify the contribution to surface area by the PS ribbons, I normalized the total surface area of PS ribbons by dividing the total PS ribbons areas (as calculated by the above method) with the bottom area of each cell culture dish/flask. The calculated PS surface areas were used to estimate the cell density. To estimate the cell density, I count the number of live cells on the surface of the PS ribbons (*N*) that I collected from each dish/flask, measured the surface area of the collected PS ribbons (*a)*, and calculate the average cells density (*n*) on the ribbons by using the equation n = N/a. The total number of cells among the ribbons (N_{total}) was then estimated as N_{total} = n = 2A.

Table 1. Calculation of the Weight of the PS Ribbons per cm^2 and Area Ratio

	Weight of PS Ribbons per dish bottom area **[W(g)/Dish area(cm^2)]**	**Ratio between the areas of PS ribbons & dish bottom** **[A_{fibers} (cm^2)/A_{dish}(cm^2)]**
Dish-1	0.017	3.22
Dish-2	0.004	0.78
Dish-3	0.004	0.70
Dish-4	0.011	2.20
Dish-5		
- Initial PS ribbons	0.004	0.70
- Adding new PS ribbons (1st time)	0.009 (=0.004 + 0.005)	1.75
- Adding new PS ribbons (2nd time)	0.014 (=0.009 + 0.005)	2.72
- Adding new PS ribbons (3rd time)	0.018 (=0.014 + 0.004)	3.50
- Adding new PS ribbons (4th time)	0.024 (=0.018 + 0.006)	4.48

2.7. Adding New PS Fibers/Ribbons to the Culturing Flask

As the hMSCs on the PS ribbons reached confluency, to provide more surface area for cell attachment and proliferation, new ribbons were added to the cell culture dishes. The PS fibers/ribbons were then briefly stirred once for each day to enhance the distribution of newly divided hMSCs among the newly added PS fibers/ribbons. In contrast to the ribbons-based group, the hMSCs culture in 2D were trypsinized upon confluency and re-cultured by the same initial cell density (7,000 cells per cm^2).

2.8. Assessment of the Differentiation Potential of hMSC

2.8.1. Evaluating the Loss of Stem cell Stemness due to Passaging

Continual re-plating can decrease of the stemness of the hMSCs. To evaluate this effect, I conducted osteogenic and adipogenic assays to evaluate the capability of the hMSC in producing bone and fat tissue, respectively. Media to induce osteogenic differentiation (bone-forming) and adipogenic differentiation (fat- forming) were applied to the hMSC expanded from 2D-cultured groups (on the P-6 and P-9) using the following protocols.

Osteogenic inductive media was supplemented with 100 nM dexamethasone (Sigma), 200 μM ascorbic acid (Sigma) and 10 mM glycerol 2- phosphate (Sigma). Adipogenic inductive media was supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 100 nM dexamethasone, 10 μg/ml insulin (Sigma), and 10 μM indomethacin (Sigma). hMSCs were cultured in the inductive media for 7 days; the medium was changed every 2-3 days.

2.8.2. Staining Assays

To evaluate the level of bone-like differentiation, hMSCs were stained for alkaline phosphatase (ALP), an osteogenic marker, using a Millipore ALP detection kit (SCR004; Millipore) per the manufacturer's instructions. Briefly, cells were fixed in 4% paraformaldehyde (PFA) for 2 minutes at room temperature, rinsed in PBS, then incubated in staining solution composed of a 2:1:1 ratio of Fast Red Violet, naphthol AS-BI phosphate, and DI H2O at room temperature in the dark for 15 minutes. After staining, samples were rinsed in PBS then imaged.

To evaluate the level of fat-like differentiation, hMSCs were stained for Oil Red O (ORO), an adipogenic marker. Briefly, cells were fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature, rinsed in PBS, incubated in 60% isopropanol for 5 minutes, then incubated in a filtered staining solution composed of 1.8 mg/ml ORO in 60% isopropanol in DI H_2O for 20 minutes. After staining, samples were rinsed in DI H_2O then imaged.

2.9. Phenotypic Characterization of hMSCs by Flow Cytometry

To examine whether the 3D, trypsin-free expansion of hMSC can improve the stability of phenotype of hMSC, I conducted flow cytometry to analyze the presence of several surface proteins that were known as the labels for hMSCs or non-hMSCs. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has defined the minimal criteria for human MSC [27]. According to thus definition, hMSC should express CD105, CD73 and CD90, but should not present CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II surface molecules.

Among the hMSC-positive marker, surface molecule CD105 takes important role in TGF-beta signalling while MSC cells are differentiating to chondrogenic cells [28]. CD73 is an important surface molecule that has functions on being involved in the bone marrow stromal interactions [29], cell migration [30], and adaptive immunity's MSC modulation [31]. The function of CD90 is less well-defined but is known to mediates the cell-cell interactions [32, 33], the adhesion of leukocytes and monocytes for fibroblasts endothelial cells [34].

As MSC-negative markers, CD14 surface molecule has some functions on monocytes and macrophages [35]. CD45 surface molecules has many functions on hematopoietic cell activation and differentiation of the cells [36]. CD34 surface molecule is a classical marker for HSCs. Its function has not defined well, according to a study CD34 has function on cell adhesion and HSC differentiation [37]. CD11b surface molecule regulates leukocyte adhesion and migration. This is important for the inflammatory response [38]. CD79a surface receptor has functional roles in B cell development and function [39]. CD19 surface molecule is a biomarker for follicular dendritic cells as well as normal B cells [40]. Finally, HLA class II surface molecules helps presenting antigens to T-lymphocytes [41].

Human MSC Analysis Kit (BD Stemflow^{TM}) (Table-2) provides a user-friendly kit for such analysis. Using this kit, I characterized the phenotype of cells expanded trypsin-free on PS Ribbons and the cells expanded with repeated trypsin treatment.

On day 20 hMSC (initially passage-5) expanded on the PS ribbons were collected by using %0.25 Tripsin (Thermo Fisher Scientific), washed with PBS and suspended at a concentration of $1x10^6$ cells/ml in FBS. Nine tubes were used for each sample, and staining for different surface markers that are indicated in Table-2 were used following the protocol-4. The stained cells were analyzed by the Flow Cytometry Machine (BD Accuri™ C6). For the 2D control group hMSCs (initially passage-5) that were trypsinized and re-plated for three times on day 10, and 20 (upon which time the cells reached confluency) were stained and analyzed by the same protocols.

Table 2. Human MSC Analysis Kit Component

Vial	Contents	Purpose
hMSC Positive Cocktail	CD90 FITC (Clone: 5E10) CD105 PerCP-Cy5.5 (Clone: 266) CD73 APC (Clone: AD2)	Cocktail to positively identify hMSCs
hMSC Positive Isotype Control Cocktail	mIgG1, κ FITC (Clone: X40) mIgG1, κ PerCP-Cy5.5 (Clone: X40) mIgG1, κ APC (Clone: X40)	Corresponding Isotype Control for hMSC Positive Cocktail
PE hMSC Negative Cocktail	CD34 PE (Clone:581) CD11b PE (Clone: ICRF44) CD19 PE (Clone: HIB19) CD45 PE (Clone: HI30) HLA-DR PE (Clone: G46-6)	Cocktail to identify potential contaminants
PE hMSC Negative Isotype Control Cocktail	mIgG1, κ PE (Clone: X40) mIgG2a, κ PE (Clone:G155-178)	Corresponding isotype control for PE hMSC Negative Cocktail
FITC Mouse Anti-human CD90	CD90 FITC (Clone: 5E10)	Compensation control
PE Mouse Anti-Human CD44	CD44 PE (Clone: G44-26)	Compensation control/MSC positive drop-in
PerCP-Cy™5.5 Mouse Anti-Human CD105	CD105 PerCP-Cy™5.5(Clone: 266)	Compensation control
APC Mouse Anti-Human CD73	CD73 APC (Clone:AD2)	Compensation control
PE Mouse IgG2b, κ Isotype Control	mIgG2b, κ (Clone: 27-35)	Corresponding Isotype Control for PE Mouse Anti-Human CD44, when used as a drop in

3. EXPERIMENTAL RESULTS

3.1. PS Ribbons Supported Cell Growth While the PS Fibers Did Not

The results from the cell culture studies showed that the PS ribbons, which were manufactured by the direct cutting of PS thin films, supported the adhesion, proliferation, and the confluency of hMSCs. 20 days after the seeding of P-5 MSC (at 7,000 cells per cm^2 of flask bottom), the cell density of MSC on the ribbons became approximately 10,000 and 20,000 cells per cm^2 (Figure 9a). The pace of cell proliferation on the PS ribbons was further improved by using surface acid treatment and gelatin coating. In contrast, the PS fibers, which were manufactured by the thermal spinning using a cotton-candy machine, did not support cell adhesion and proliferation (Figure 9b). 20 days after the seeding of P-5 MSC (at 7,000 cells per cm^2 of flask bottom), the cell density of MSC on the fibers became approximately 100 to 2,000 cell per cm^2. The number live hMSCs among the PS fibers gradually decreased over the course of three weeks, as showed by the weakening Calcein-AM signals.

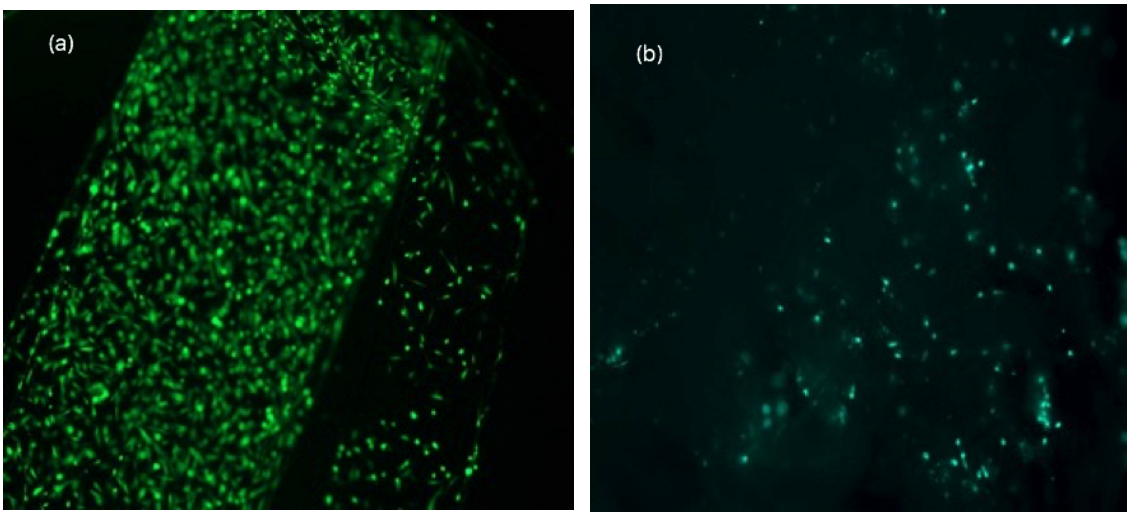


Figure 9. Adhesion and proliferation of hMSC on day 20 on the a) PS Ribbons and b) PS Fibers (Calcien-AM Staining)

3.1.2. The Lower Initial Amount of PS Ribbons Enhance the Initial Rate of all Proliferation

The initial proliferation of the cell on the PS ribbons was found much slower than the rate of proliferation of hMSCs on 2D culture. In the traditional 2D culture, the hMSC often reached confluency on the 10^{th} day post cell seeding, and the cell proliferation rate on day 10^{th} was 3 to 4 folds (Figure 10). In contrast, in my initial trial (Figure 11a) the proliferation rate of MSC on the PS fibers barely changed. The density of MSC on the fibers was between 400 and 3000 cell density per cm^2 from day 1 to day 45. I found that this was due to the fact that the initial amount of PS ribbons was too high (0.017 per cm^2 of small culture plate), which led to an extremely low initial cell density (<3,000 cells per cm^2) on the surface of the PS ribbons.

In my next trial (Figure 11b), I used a smaller amount of PS ribbons (0.004 g per cm^2 of culture plate) to start the 3D culture, which led to a higher cell density on day 21(about 10,000 cells per cm^2). Decreasing the initial amount of PS ribbons successfully increased the cell proliferation rate and the cell become 5.33-fold by day 20. However, the cell density raised to 5.33-fold the original cell density by day 20^{th}. Such cell density, however, plateaued on day 20^{th} and did not changed statistically beyond day 20^{th}. Repeating the same experimental setting once led to the similar result (Figure 11c).

3.1.3. Stirring the Ribbons Enhances the Speed of 3D Cell Proliferation

Next I tested whether regular stirring of the PS ribbons, which constantly exposed new surfaces for cells adhesion could further enhance the proliferation of hMSCs. In my third trial (Figure 11d), the PS fibers/ribbons were briefly stirred once each day to enhance the distribution of newly divided hMSCs among the PS fibers/ribbons with 7.84-fold on day 20. Surprisingly, the hMSCs among the regularly stirred PS ribbons proliferated to up to 9 folds within 2 weeks.

3.1.4. Adding new ribbons enabled the MSC to continually proliferate

The cell density of group in Figure 11d plateaued on day 16th and did not changed statistically for the following days. I deduced that the cells had approached confluency among the PS ribbons; the available PS surface for cell expansion might be depleted. To learn whether increasing the available PS surface could further increase the cell proliferation, in the new batch of cell culture (dish-5) I gradually added new PS ribbons (by 0.0128 g per cm^2 of culture plate) to the culturing flasks (Figure 11e), thereby gradually increasing the total amount of surface area for cell adhesion. The distribution of newly divided hMSCs among the newly added PS fibers/ribbons was enhanced by the brief stirring of ribbons (one time each day).

New ribbons were added to the culture flasks on day 6, day 16, 22 and 38. The addition of new PS ribbons caused an initial drop to the estimated number of hMSCs, because the newly added ribbons, which hosted much less cells than the old ribbons, were being collected together with the old ribbons for cell counting. The estimated cell numbers recovered 2 to 6 days after the initial drop and continued to increase, as the hMSCs among the new ribbons proliferated toward confluency again.

Of particular interest is the effect of the gradual increase of PS ribbons density (g per cm^2 of flask area) on enhancing the proliferation rate of hMSCs. In dish-5, increasing the amount of PS ribbons gradually from a low initial ribbons density to a high density, such as from 0.70 to 4.48 g per cm^2 of flask area in dish-5, increased the total number of hMSCs by 13.61 folds in 20 days (Table 3). In contrast, culturing hMSCs by a high initial ribbons density, such as 3.22 g per cm^2 of flask area in dish-1, led to an extremely low cell proliferation rate on day 20.

In my 3D platform, it is worth noticing that, while the **initial** amount (or density) of PS ribbons determine the initial cell proliferation speed, it is the **final** amount (or density) of PS ribbons that determines the ribbons' maximum capacity for cell expansion: the final amount ribbons determines how much surface area in total is available for cell attachment and proliferation.

However, gradually adding new PS ribbons didn't affect the proliferation rate of the PS ribbons until the cells reached confluency. The increased proliferation in dish-5 appeared

to be result of the increased cell density of PS ribbons. On the other hand, when the cells reached confluency on the dish-4, there wasn't enough surface area to continue increasing of proliferation on the cell culture. Gradually adding PS ribbons (dish-5) helped to maintain the stability of increasing cell proliferation as limitless. Since, surface area increased when the cell reached the confluency.

Table 3. Calculation of the Fold Change on the PS Ribbons on day 20

	Weight of PS Ribbons per dish bottom area **[W(g)/Dish area(cm^2)]**	**Ratio between the areas of PS ribbons & dish bottom** **[A_{fibers} (cm^2)/A_{dish}(cm^2)]**	**Calculated Fold Change on Day 20**
Dish-1	0.017	3.22	**1.26 - Fold**
Dish-2	0.004	0.78	**5.33 - Fold**
Dish-3	0.004	0.70	**4.24 - Fold**
Dish-4	0.011	2.20	**7.87 - Fold**
Dish-5			
- Initial PS ribbons	0.004	0.70	**13.61 - Fold**
- Adding new PS ribbons (1st time)	0.009 (=0.004 + 0.005)	1.75	
- Adding new PS ribbons (2nd time)	0.014 (=0.009 + 0.005)	2.72	
- Adding new PS ribbons (3rd time)	0.018 (=0.014 + 0.004)	3.50	
- Adding new PS ribbons (4th time)	0.024 (=0.018 + 0.006)	4.48	

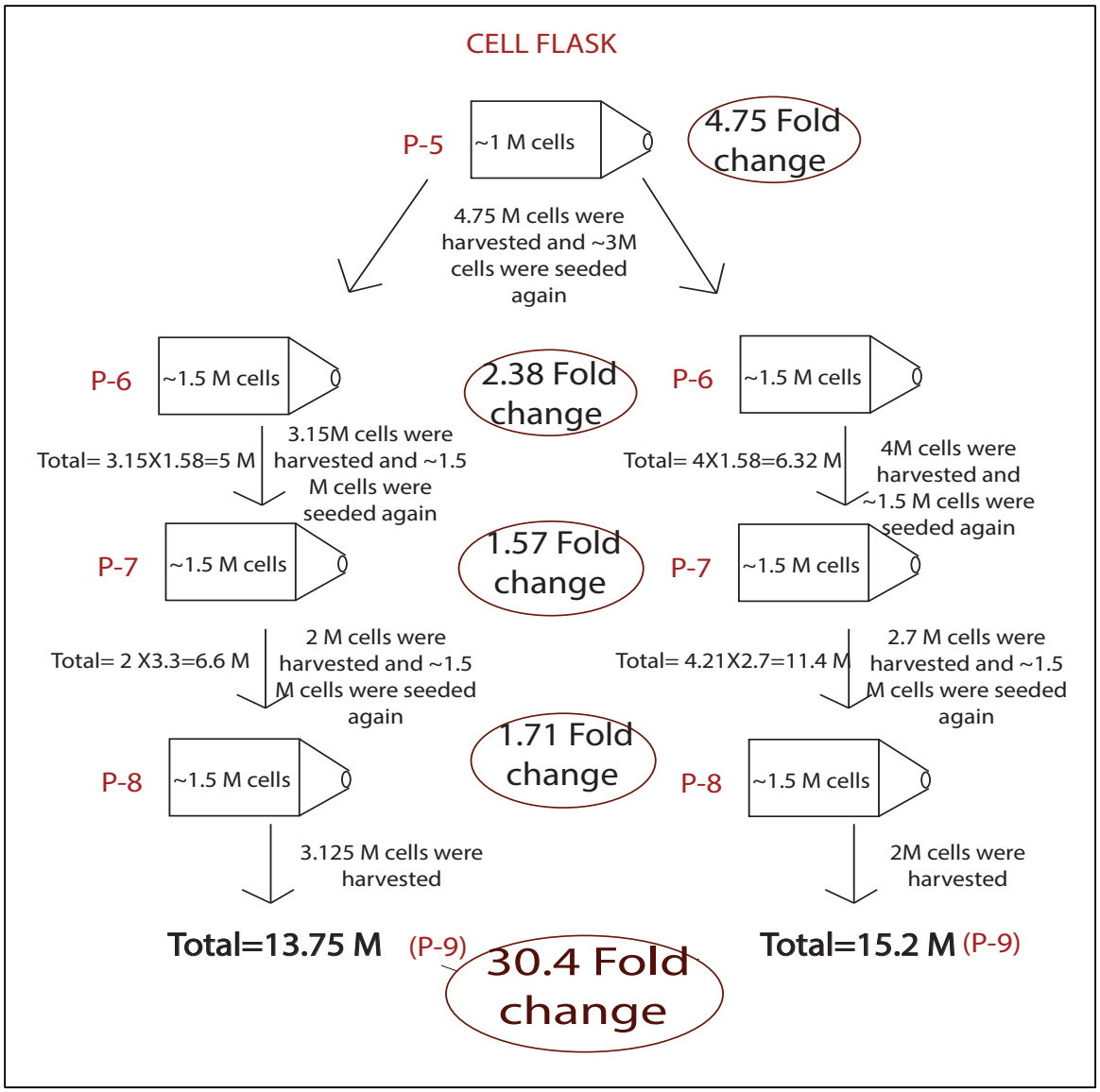


Figure 10. Cell passaging with traditional 2D culture. The hMSC on the 2D culture flask was cultured until confluency, detached, and re-plated for three times

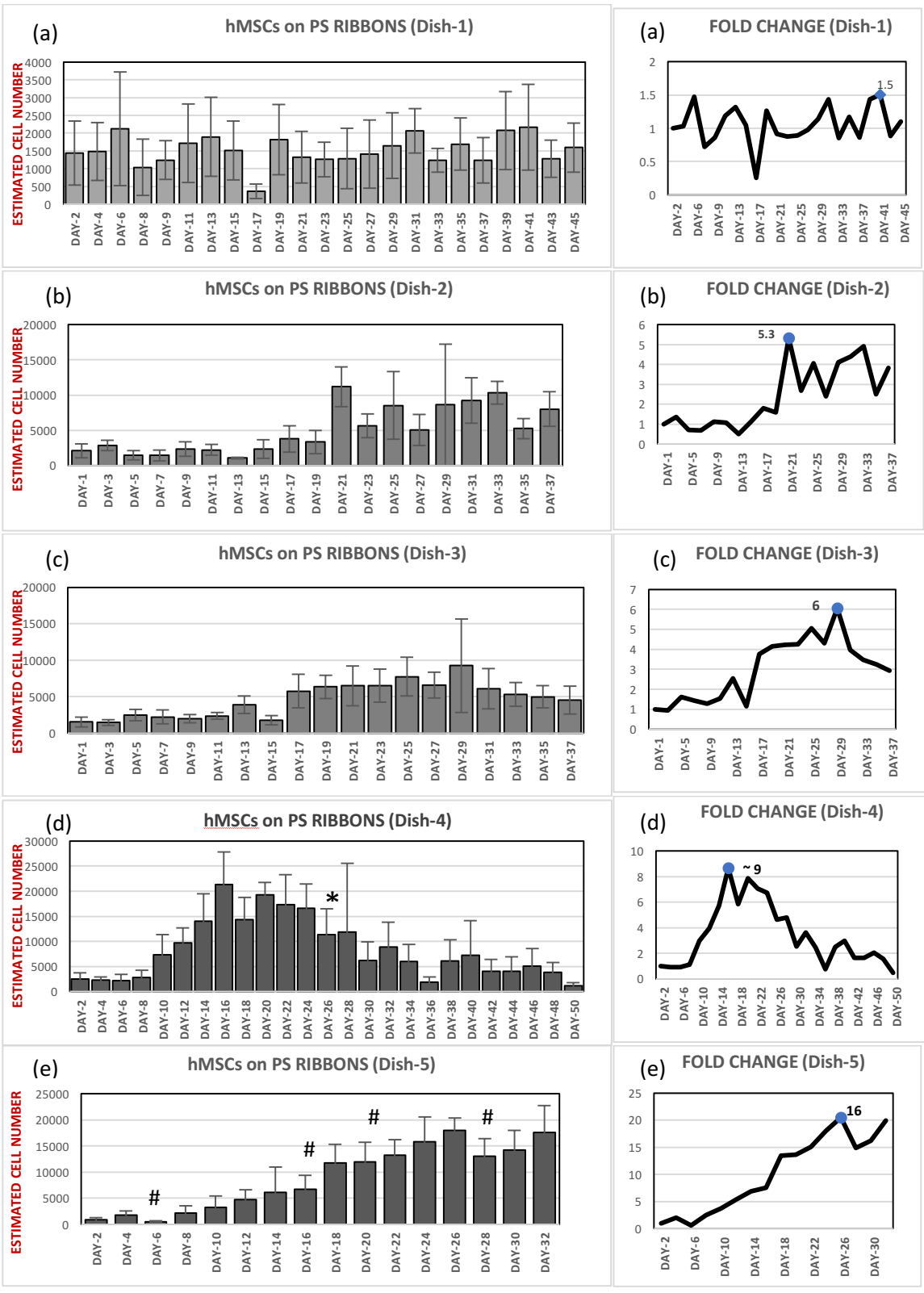

(a)
hMSCs on PS RIBBONS (Dish-1)
ESTIMATED CELL NUMBER
(a)
FOLD CHANGE (Dish-1)
1.5
(b)
hMSCs on PS RIBBONS (Dish-2)
ESTIMATED CELL NUMBER
(b)
FOLD CHANGE (Dish-2)
5.3
(c)
hMSCs on PS RIBBONS (Dish-3)
ESTIMATED CELL NUMBER
(c)
FOLD CHANGE (Dish-3)
6
(d)
hMSCs on PS RIBBONS (Dish-4)
ESTIMATED CELL NUMBER
*
(d)
FOLD CHANGE (Dish-4)
~ 9
(e)
hMSCs on PS RIBBONS (Dish-5)
ESTIMATED CELL NUMBER
#
(e)
FOLD CHANGE (Dish-5)
16

Figure 11. The proliferation rate of hMSCs among the PS ribbons was highly sensitive to (i) the initial amount of PS ribbons (a vs. b & c), (ii) the stirring of PS ribbons (d), which exposes new binding surface for cell attachment, and (iii) the addition of new PS ribbons upon confluency (e), which provided new surface area for cell adhesion and expansion. (*) Changing the petri dish. (#) Adding new part PS ribbons to flask.

The differences between the traditional, 2D cell expansion and ribbon-based, 3D cell expansion was shown on the Table-4. According to results, the cells cultured by the 2D traditional methods reached 17.7-fold within 35 days. The cells cultured by the 3D traditional methods reached 20-fold within 25 days. The fold change on the traditional group was faster than the fold change on ribbon-based group within first 2 week. However, ribbon-based group can maintain the fold change in a higher point (20-fold). These results suggested that 3D ribbon based group improved the fold change with a higher cell density.

Table 4. The Comparison Between 2D Traditional and 3D Ribbon-Based Method (*=lack of data because of contamination)

Time Points	2D TRADITIONAL METHOD Fold Change	3D RIBBON BASED METHOD Total Fold Change	Accumulated FOLD CHANGE	
			2D	3D
Day 0	1M cell seeded (initial fold change)	1M cell seeded (initial fold change)	-	-
1st Time point	1st Passaging, 4.8 fold (Day 10th)	1.5 Fold Day (10th)	4.8 Fold	1.5 Fold
2nd Time Point	2nd Passaging, 2.4 fold (Day 25th)	20.4 Fold (Day 25th)	11.3 Fold	20.4 Fold
3rd Time Point	3rd Passaging, 1.6 fold (Day 35th)	20 Fold (Day 33th)	17.7 Fold	20 Fold
Final Time point	4th Passaging, 1.7 fold (Day 45th)	*	30.4 Fold	*

3.2. Effects of cell passaging on the Stemness of hMSCs

The differentiation potential of the human mesenchymal cells was evaluated by stimulating the cells with growth factors that promote adipogenic (fat producing) or osteogenic (bone producing) differentiation. P-5 hMSCs expanded for 10 days and 1 passage in traditional cell flask (P-6 cells) and hMSCs expanded for 40 days and four passages by traditional two-dimensional cell culture (P-9 cells) were re-plated in 2D in a 24-well plate, cultured in hMSC media for 24 hours, and exposed to the osteogenic or adipogenic differentiation media. All samples were incubated at 37 C in 5% CO_2 for one week and stained for alkaline phosphatase (ALP), an osteogenic marker, and stained with Oil Red O (ORO), which labels fatty acid components.

3.2.1. P-6 hMSCs showed highly selective bone and fat-like differentiation

For the P-6 hMSCs, which were only treated by one time for cell detachment and trypsinization, results from the differentiation assay showed obvious difference between the osteogenic medium-treated and adipogenic medium-treated groups (Figure 12 vs. 14). The ALP staining, which label bone tissues, was strongly visible in the osteogenic medium-treated group (43.78%) and was almost absent in the adipogenic medium-treated group (0.73%) (Figure 12, 14a vs. 14d). In contrast, the ARO staining, which labels fat tissues, was visible in the adipogenic medium-treated group (4.6%) but less visible in the osteogenic medium-treated group (3.98%) (Figure 12, 14b vs. 14e). Interestingly, the hMSCs cultured for one week without any growth factor showed a trace amount of ALP (bone tissue) signals (10.71%) (Figure 12, 14g). This implies that without adipogenic induction, which is known to suppress bone differentiation, the hMSCs tend to creep toward the phenotype of bones.

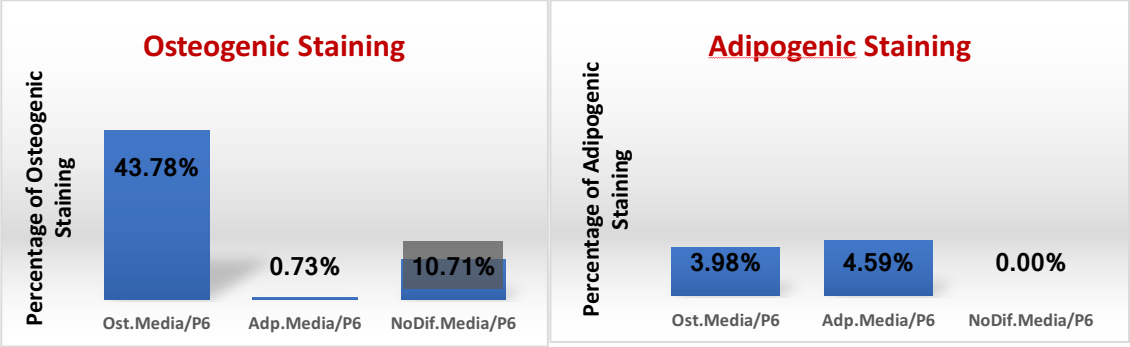


Figure 12. Percentages of P-6 cell staining by using image J program a) osteogenic(ALP) staining b) adipogenic(ARO) staining

3.2.2. P-9 hMSCs showed sigh of bone differentiation without induction

For the P-9 hMSCs, which had exposed the hMSC four times to cell detachment and trypsinization, the results from the assays demonstrated that the differentiation of hMSCs had a strong tendency toward osteogenic differentiation, regardless of which type of growth factors were used (Figure 13, 15). The bone-tissue staining was strongly visible in the osteogenic medium-treated group (45%), and was also visible in the adipogenic medium-treated group with 14.55% percentage (Figure 13, 15a vs. 15d). In contrast, the fat tissues staining was weakly visible in the adipogenic medium-treated group (2.9%) but absent in the osteogenic medium-treated group (Figure 13, 15b vs. 15e). P-9 hMSCs that were cultured without growth factors showed a stronger amount bone tissue signals (20.25%) than the P-6 hMSCs without growth factors (Figure 15g vs. Figure 15e).

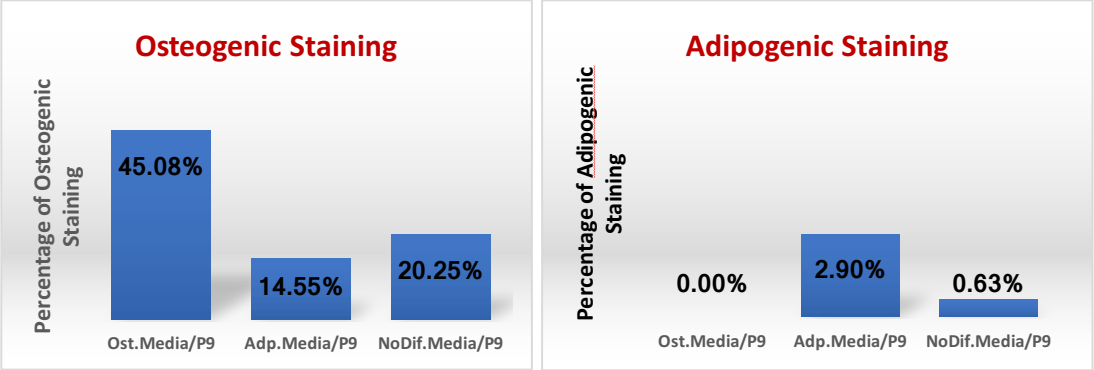


Figure 13. Percentages of P-9 cell staining by using imageJ program a) osteogenic staining b) adipogenic staining

3.2.3 Cell Passaging decreased the differentiation potential of hMSCs

The above results indicated that the decreasing trypsinization and replanting during the cell expansion helped maintained the differentiation potential of hMSC. In contrast, the increased passage number on cell expansion, which required multiple times of trypsinization and replanting, decreased the differentiation potential of hMSC, promoting bone tissue differentiation while inhibiting fat tissue differentiation.

To understand if the cell expansion in 3D environment created by PS ribbons can improve the stability of MSC phenotype, I next conducted flow cytometry to characterize the surface markers of hMSCs, to learn whether the different types of cells expansion had induced different types of gene expression in the hMSCs.

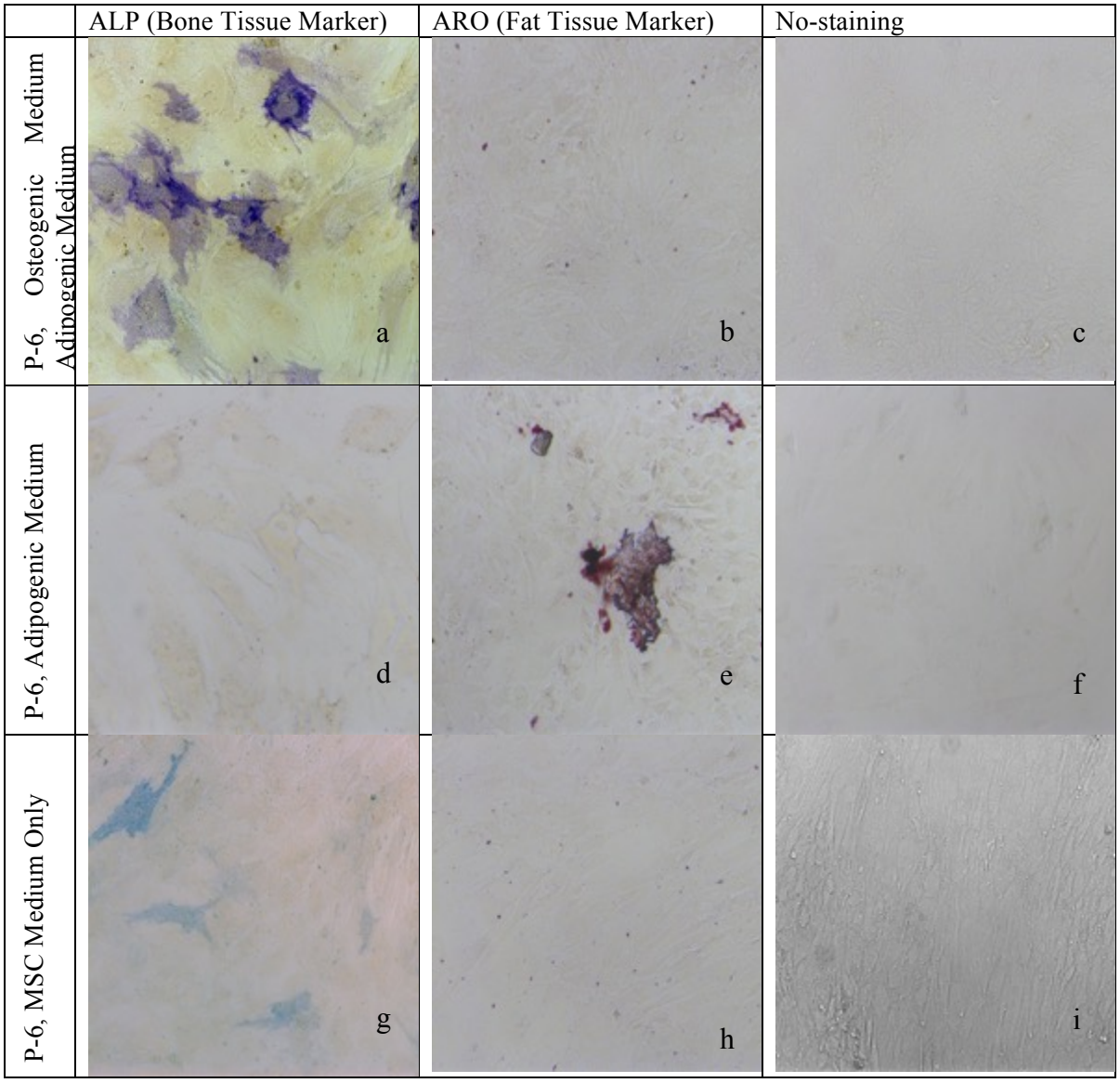


Figure 14. Differentiation assay for PS ribbons-expanded hMSCs (P-6 cells)

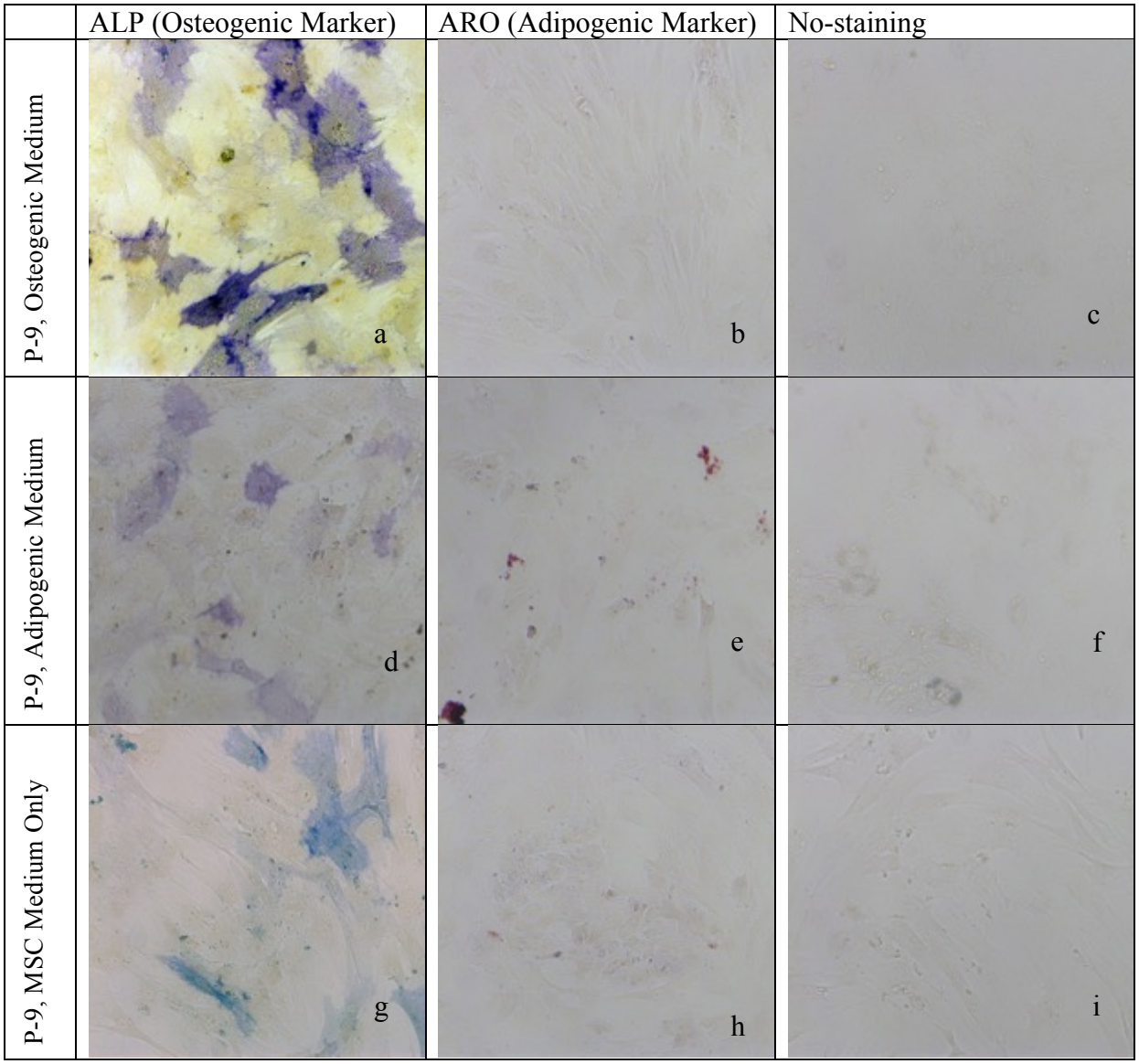


Figure 15. Differentiation assay for 2D-expanded hMSCs (P-9 cells)

3.3. Phenotypic Characterization of hMSCs by Flow Cytometry

Flow cytometer (BD Accuri™ C6) was used to measure the levels of expression of the surface proteins that represent the phenotype of MSC. These marker proteins were suggested by The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy. I selected a flow cytometry kit (BD Stemflow™) that labels both the positive and negative cell surface antigen and applied the kit to the hMSCs that were expanded by the PS ribbons (10 days, 5 passages) and the hMSCs expanded by the traditional tissue-culture plates (20 days, 5 passages). The hMSCs were collected as two different group on the 10^{th} and 20^{th} day respectively after cell seeding (Figure 17a, 18a). The MSC-positive marker proteins I selected were CD90, CD105, and CD73, and the MSC-negative markers I selected was CD44, as well as a cocktail of negative markers including CD34, CD11b, CD19, CD45, and HLA-DR.

I used two different groups to evaluate how the methods of cell expansion (3D vs. 2D) affects the cells' phenotype post expanding. For the first group, cell expanded by the PS ribbons were collected on the 10^{th} day after cell seeding (Figure 17). 0.25% Tripsin was used to detach the cells. For the second group, cell expanded in 2D by the traditional cell culture dishes/flasks were collected on the 20th day after Passage-5 cell seeding (Figure 18). 0.25% Tripsin was used as a detachment solution too.

Using isotype control as background, the flow cytometry results suggest that all the samples contain a significant number of non-hMSCs as defined by the minimal criteria for MSC surface's antigen phenotyping. CD44 marker demonstrated negative expression as 95.43% positive (PS ribbon group) (Figure 17b) and 82% positive (2D-expanded group) (Figure 18b). CD73 markers demonstrated positive expression as 93.21% (PS ribbon groups) (Figure 17e) and 98.06% (2D-expanded group) (Figure 18e) positive. However, for both the 3D and 2D groups, the signals of positive markers CD105 and CD90 positive makers (on the both groups) were bleeding to Isotype control (Figure 17c,d and 15c,d), suggesting that many cells from each group did not express CD 105 and CD90 (which should be expressed in MSC). In addition, there is no negative expression

of all markers included in the negative cocktail (CD34, CD11b, CD19, CD45, HLA-DR) (Figure 17f and 18f). Therefore, these markers (negative cocktail, CD105 and CD90 positive markers) didn't ensure the criteria of the certain surface markers to be hMSC. The above results show that in both group a large cell population was already losing the MSC phenotype. The above results showed no sharp phenotypic difference between the 3D, PS ribbon-expended group's cells and the 2D-expanded group's cells.

The results from the above flow cytometry assay and the results from the osteogenic/adipogenic differentiation assay, which showed that the passage-5 MSCs that I used tended to differentiate toward bone-like cells, showed that the MSC that I used were too high in passage and had already lost the MSC phenotype before the expansion. The above results are therefore insufficient to conclude whether the PS ribbons-assisted cell expansion can better maintain the phenotype of hMSC.

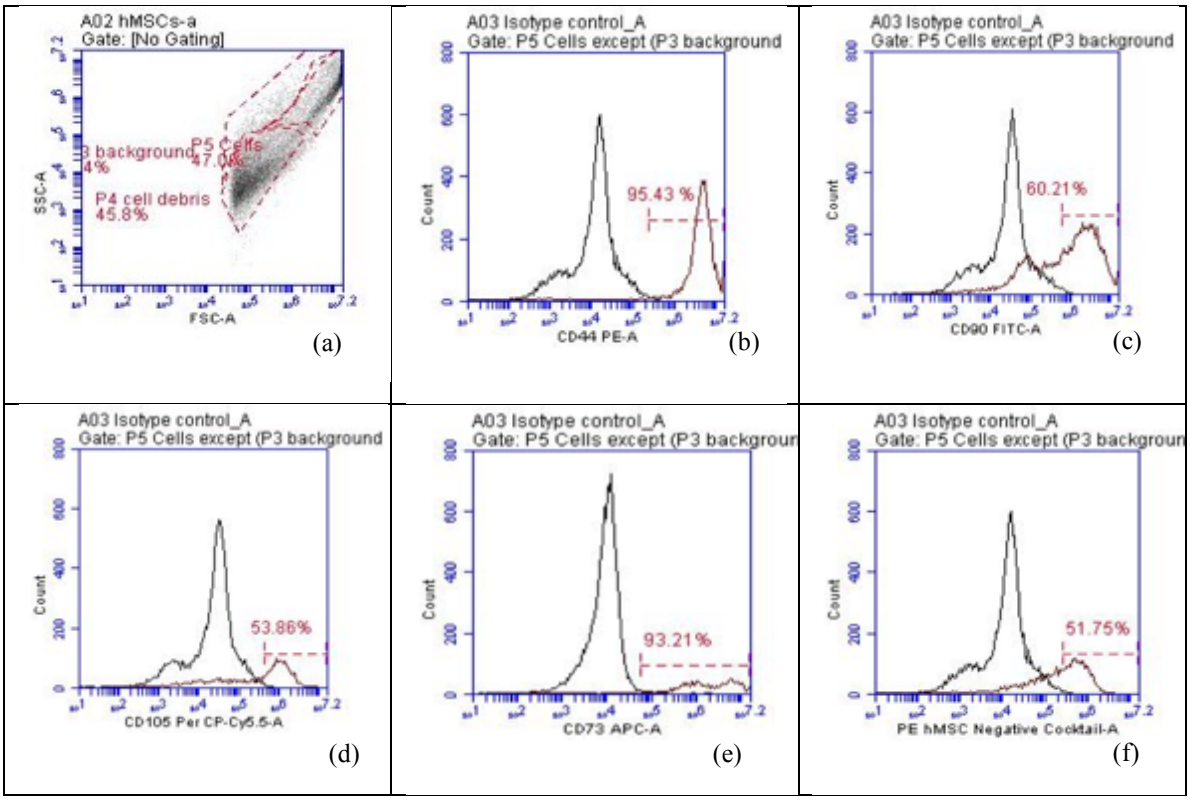


Figure 16. Analyses of positive and negative cell surface antigen in an expansion of bone-marrow hMSCs (collected from PS-Ribbon group on the 10th day after Passage 5 cell seeding). a) Representing the cell on the plot graph. Isotype Control (black lines), b) CD44 negative marker (red line), c) CD90 Positive Marker (red line), d) CD105 positive marker (red line), e) CD73 positive marker (red line), f) hMSCs negative cocktail (red line)

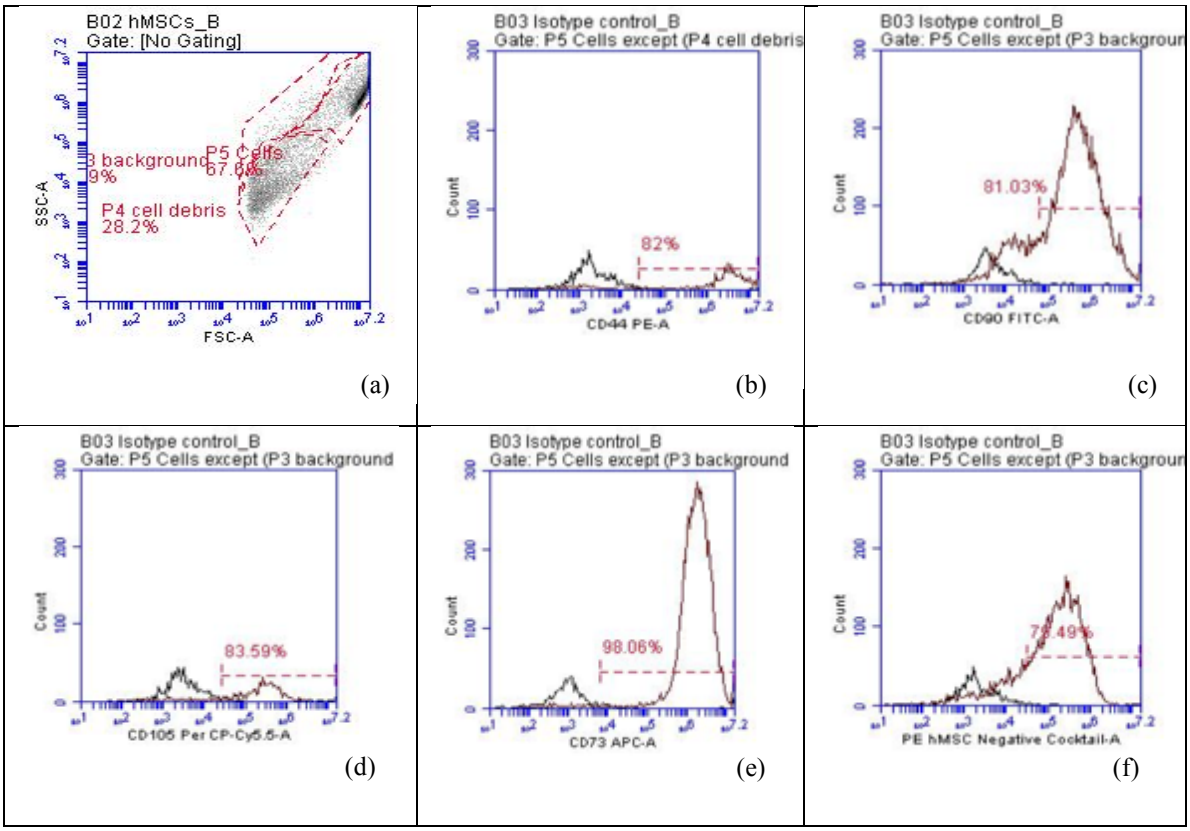


Figure 17. Analyses of positive and negative cell surface antigen in an expansion of bone-marrow hMSCs (collected from 2D-expanded group on the 20th day after Passage 5 cell seeding). a) Representing the cell on the plot graph. Isotype Control (black lines), b) CD44 negative marker (red line), c) CD90 Positive Marker (red line), d) CD105 positive marker (red line), e) CD73 positive marker (red line), f) hMSCs negative cocktail (red line)

4. CONCLUSION

In conclusion, my aims for this Master research have been met by the following results:

Fibers and ribbons-shape PS substrates were fabricated using different type of fabrication methods. The thermally spun, fiber-shape substrates were shown ineffective for cell-culture, which was probably caused by the machine heat during the thermal spinning. In contrast, the PS Ribbons made by thin-film cutting were efficient in supporting cell adhesion, survival, and expansion. A 3D cell-expansion platform was created with using PS Ribbons. The results of Dish-5 ribbon-based group (3D) demonstrated that the PS ribbons provided a cell proliferation in the capacity (> 8-fold) that is higher than the traditional cell expansion (3 to 5 fold) within 20 day after cell seeding. The expansion of cells in 3D was enabled by both the regular stirring of PS ribbons and increasing the cell density of PS ribbons. Gradually adding new PS ribbons helped increasing of the surface area, while the stirring facilitates the distribution of newly born cells among the PS ribbons whenever the cells reached confluency.

Among the PS-ribbons, the speed of cell proliferation was initially (during 2 weeks) shown to be much slower than the traditional (reaching the confluency within 2 weeks) 2D cell expansion. However, the speed of cell proliferation among the PS-ribbons was later accelerated by improving cell-expansion protocol; the improvement includes controlling the initial amount of PS ribbons, stirring the PS ribbons daily into the cell culture flask.

The differentiation assay results showed that both P-6 and P-9 hMSCs have a strong tendency toward osteogenic differentiation. However, the P-6 hMSCs expanded by the traditional 2D cell culture, which received 1 time re-plating treatment (trypsinization) during the expansion, showed higher potential for fat-like cells production in comparison with P-9 hMSCs expanded by the traditional, 2D cell culture, which received 4 times re-plating treatments during the expansion. Such results indicated that the decreasing trypsinization and re-planting the cells during the cell expansion helped maintained the differentiation potential of hMSC. Cell passaging decreases the stemness of the hMSCs and multipotentiality.

Immuno-phenotyping did not show that the expansion by PS-ribbons better promotes MSC phenotype. Both 3D and 2D expanded groups gave the similar results with the differentiation assay that the hMSCs I used were probably too high in passage (P-5) and had already aged. The flow cytometry results showed that both PS ribbon cell and 2D-expanded cells presented CD73 positive surface marker and not present CD44 negative surface marker in maintaining the MSC phenotypes, but they were not real hMSCs based on the negative surface markers' (negative cocktail) and, CD90 and CD105 positive surface markers' expressions. According to flow cytometry result I cannot say the cells used in the experiment were hMSCs.

5. FUTURE WORKS

Despite the potential that I found in 3D cell expansion, the results from the immuno-phenotyping assay was insufficient to conclude whether the PS ribbons-assisted cell expansion can better maintain the phenotype of hMSCs. To answer this question, I will continue this project as follows:

1. Younger hMSCs, such passage-2, will be used for the cell expansion to prevent cell senescence as early and distinguish early differentiation from the sample.
2. Differentiation test for all 3D samples with their control will be conducted to understand the differentiation potential between 3D and 2D cell culture groups.
3. Passage number might be effect the all statistical result. Therefore, the statistical analysis with cells on the different Passage will be monitored and the cell density per cm^2 and the fold change will be calculated by using image J program with essential control groups.
4. The effect of MSC aging could be caused by either proliferation, by re-plating, or by both. To know the answer, the differentiation and flow cytometry assays should be done again with MSCs that are expanded by the same amount of fold.

6. PROTOCOLS

6.1. Protocol for Preparing Fiber with Using Cotton Candy Machine (Protocol-1)

Getting the best polystyrene (PS) powder is depend on the amount of PS into the machine. 0.5, 1.0, 2.0, 4.0 g PS were used to test that which one is the best option to create a perfect fiber without junk. 0.5 g PS showed the least junk and junk population had increased with increasing PS amount.

Step-1. Open candy machine, turn on the heat mode and wait 15 minutes to be ready for using (pre-heat).

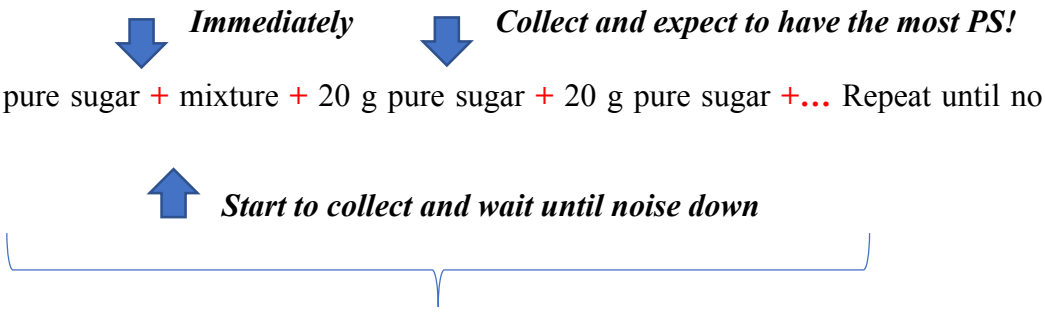
Step-2. Prepare a mixture to with using 10 g sugar and 1 g PS into a 50-ml tube. Make sure to mix it.

Step-3. Add the approximately 30-40 g sugar into the machine nest. Immediately, add mixture on the same place. Pick the fibers up from the machine and put the water. Wait until sugar be dissolved. This time, you cannot see the fiber clearly.

Step-5. Wait until the machine's noise goes down and fiber producing is stopped, and then add 11 g sugar + PS Powder more. And pick them up and in the same water solve it again. This time you can see the fibers clearly.

Step-6. Repeat the process until you cannot see any fiber on the water. Every time, you add 20 g sugar and pick up and solve it with the water.

Step-7. Your machine is ready and enough clear now and you can use it for the other experiment safely later.



! On the normal condition, more PS needs more steps to collect all until overload point for the machine. That's why the machine cannot continue to produce PS fiber correctly without junk.

In the experiment, to make **mixture**;

Sucrose	***Polystyrene (PS)***
20 g	*0.5 g*
20 g	*1.0 g*
20 g	*2.0 g*
20 g	*4.0 g*

The best option is (0.5+20) g mixture because it doesn't have almost any junk. In our experiment, we added (+20) g sugar for the mixture included

6.2. Preparing MSC Culture Media (Protocol-2)

1. media 500mL
2. (10% FBS)1 tube of FBS 45ml in purple
3. (1% P/S)1 tube small blue PS 5ml
4. growth factor bFGF, 1 tube, in square box

6.3. Calcien-AM Live-Cell Staining Protocol (Protocol-3)

1. Prepare 1 ml nuclear dye staining solution (1:1000 dilution, stored in -20).
2. Into a 50-ml tube, prepare a mixture with using 1 microliter staining solution (Calcein-AM) and 1 ml PBS. Make sure pipet it enough to ensure homogeneity.
3. From your sample, collect some sample cut. Make sure to collect a thin layer to observe more clearly.
4. Clean culture media from your cells with using 40 ml PBS. Repeat it 2 times.
5. Add the mixture (Calcein-AM+PBS) into the sample cut. Make sure to flatten the sample cut with mixture.

Incubate for 10 minutes under 37 ^{o}C, and monitor the sample under the microscope. Sample should be inside in the mixture solution by all angles, sample cut should be surrounded by the mixture solution. To ensure it, place the plate into the incubator with an angle.

6.4. Flow Cytometry Protocol (Protocol-4)

Recommended Assay Procedure (Given by BD Biosciences):

1. Detach cells using Cell Detachment Solution (%0.25 trypsin), wash cells and resuspend at a concentration of 1x10^6 cells/ml in the staining buffer (FBS).
2. Label tubes and add antibodies as shown below:

Tube Add (1 test size);

(1) FITC Mouse Anti-Human CD90 (5µl)

(2) PE Mouse Anti-Human CD44 (5µl)

(3) PerCP-CyTM5.5 Mouse Anti-Human CD105 (5µl)

(4) APC Mouse Anti-Human CD73 (5µl)

(5) Nothing

(6) hMSC Positive Isotype Control Cocktail (20µl) PE hMSC Negative Isotype Control Cocktail (20µl)

(7) hMSC Positive Cocktail (20µl) PE hMSC Negative Cocktail (20µl) And/or

(8) hMSC Positive Isotype Control Cocktail (20µl) Drop in isotype control (i.e. PE Mouse IgG2b, κ) (5 µl)

(9) hMSC Positive Cocktail (20µl) PE Drop in (i.e. PE Mouse Anti-Human CD44) (5µl)

3. Repeat tubes 5-7 and/or 8-9 for each additional cell sample.
4. Add 100 µl of prepared cell suspension to tubes 1 through 9.
5. Incubate tubes in the dark for 30 minutes (May be done on ice or at room temp).

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